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Inhibition of advanced glycation end products formation by stilbene and phenanthrene derivatives from *Prosthechea michuacana* *in vitro* and *in vivo*

[Inhibición de la formación de subproductos en la glicosilación avanzada por derivados de estilbeno y fenantreno de *Prosthechea michuacana*, *in vitro* e *in vivo*]

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Abstract
In our previous study, we isolated from chloroform extract of the bulbs of orchid *P. michuacana*, three antioxidant compounds: two stilbene α-α´-dihydro, 3´,5´,2-trimethoxy-3-hydroxy-4-acetyl-4´-isopentenyl stilbene, 5-[2-(3-hydroxy-5-methoxyphenyl)ethyl]-2-methoxyphenol (gigantol) and one phenanthrene 4,6,7-trihydroxy-2-methoxy-8-(methylbut-2-enylphenanthren-1-1´-4´,6´,7´-trihydroxy-2´-methoxy-8´-(methylbut-2´-enyl)-phenanthrene. Following the study, we investigated the ability of isolated compounds to inhibit advanced glycation *in vitro*. Bovine serum albumin was glycated in the presence of glucose or methylglyoxal. Amadori-rich protein was prepared by dialyzing lysozyme that had been glycated by ribose. We also evaluated renal function by checking formation of advanced glycation and tail tendon collagen quality in streptozotocin-induced diabetic mice. Also determined the effect on LDL and hemoglobin. Compounds can efficiently inhibit the formation of AGEs by trapping reactive methylglyoxal and showed potent anti-Amadorin activity. Also exhibited a significant inhibitory activity on the glycated hemoglobin (GHb and HbA_{1c}). Compounds showed a protective renal effect and reduction in mice tail tendon collagen. Also the tested compounds are potent agents for protecting LDL against oxidation and glycation. We concluded that compounds from *P. michuacana* are potent antiglycation agents, which can be of great value in the prevention of diabetic glycation-associated-pathogenesis.

Keywords: *Prosthechea michuacana;* antiglycation; stilbenoids; phenanthrene; streptozotocin-induced diabetic mice

Resumen
En un estudio anterior, aislamos del extracto clorofórmico de los bulbos de la orquídea *Prosthechea michuacana*, tres compuestos antioxidantes: los estilbenos α-α´-dihidro, 3´,5´,2-trimethoxi-3-hidroxi-4-acetil-4´-isopentenil-stilbeno, 5-[2-(3-hydroxy-5-methoxyphenyl)ethyl]-2-methoxyphenol (gigantol) y el fenantreno 4,6,7-trihidroxi -2-methoxi-8-(metilbut-2-enilfenantren-1-1´-4´,6´,7´-trihidroxi-2´-metoxi-8´-(metilbut-2´-enil)-fenantreno. Continuando con el estudio, investigamos la capacidad de estos compuestos para inhibir la glicación avanzada *in vitro*. La seroalbúmina bovina se glicosiló en presencia de glucosa o metilglioxal. La reacción de Amadori se determinó con lisozima glicosilada previamente tratada con ribosa. También se evaluó la función renal mediante la formación de la glicación avanzada y la inhibición de AGEs en el ensayo sobre el colágeno del tendón de la cola en ratones con diabetes inducida con estreptozotocina. También determinamos el efecto de los compuestos aislados sobre LDL y hemoglobin. Los compuestos pueden inhibir eficazmente la formación de AGE atrapando el metilglioxal reactivo y muestran potente actividad anti Amadorin. También mostraron una actividad inhibitoria significativa en la formación de la hemoglobina glucosilada, GHB y HbA1c. Mostraron un efecto protector renal y una reducción en el colágeno glicosiladó del tendón de la cola. También estos compuestos son potentes agentes para la protección de LDL frente la oxidación y la glicación. En base a los resultados obtenidos se concluye que los compuestos aislados son potentes agentes antiglicación, que pueden ser de gran valor en la prevención de la patogénesis de la diabetes asociada a la glicación.

Palabras Clave: *Prosthechea michuacana;* antiglicación; estilbenoides; fenantreno; ratones con diabetes inducida por estreptozotocina

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Lista de abreviaciones: AGEs: productos finales de la glicación avanzada; BSA: albumina de sérica bovina

INTRODUCTION

Advanced glycation end-products (AGEs) are the final products of the nonenzymatic reaction between reducing sugars and amino groups in proteins, lipoproteins and nucleic acids. They are a group of complex and heterogeneous compounds that are known as brown and fluorescent cross-linking substances such as pentoside, nonfluorescent cross-linking products like methylglyoxal-lysine dimers, or nonfluorescent, non-cross-linking adducts such as carboxymethyllysine and pyrraline, a pyrrole aldehyde (Rahbar and Figarola, 2002). AGEs accumulation *in vivo* has been considered to play a major role in the pathogenic process of diabetes and its complications, including neuropathy, nephropathy, retinopathy, and cataract (Ahmed, 2005) and in other health disorder such as atherosclerosis (Kune *et al*., 1978), Alzheimer´s disease (Vitek *et al*., 1994), and aging (Brownlee, 1995). Thus, the discovery and investigation of compounds with an AGEs inhibitor activity, would certainly offer a potential therapeutic approach for the prevention of diabetes or other pathogenic complications.

Prosthechea michuacana (Lex.) W. E Higgins (PM) belong to the family Orchidaceae. Mixteco and Zapoteco natives of Oaxaca Mexico, give to this plant several uses: As food they collect the bulbs and eat them raw to eliminate thirst or liquefy them with water to prepare a drink, that is also used as anti-inflammatory, to depurate the circulatory system, treat renal disease, and diabetes (Perez, 2010). During November and December, this beautiful orchid is used for religious purposes as a decorative element in the traditional "Birth" (Flores and Manzanero, 1988). We have previously reported the relaxant and antispasmodic effect of this plant on isolated guinea pig ileum (Vargas and Perez, 2009a), hepatoprotective activity, ability to inhibit oxidative stress in liver (Perez and Vargas, 2009b; Perez and Hoyos, 2011), anti-inflammatory and wound healing properties (Perez and Vargas, 2009a), nephroprotective (Perez *et al*., 2010a) antioxidant activities (Perez *et al*., 2010b) and anti-diabetic activity (Perez and Vadillo, 2011). The aim of this study, is to investigate the AGEs inhibition capacity of two stilbenoid and one phenanthrenes previously isolated from the bulbs of *Prosthechea michuacana*.

MATERIALS AND METHODS

Reagents

All chemicals and reagents used in this study were purchased from Sigma chemical Co (St. Louis, MO, USA). All reagents used were of analytical grade. GlycoGel II column (Pierce, Rockford, IL),

General Experimental Procedures

The fluorescence was measured using Aminco Bowman Luminescence spectrometer (USA). The amount of glycated hemoglobin (% GHb) was determined using an ion capture component set (IMx system, Abbott laboratories, USA). Absorbance was recorded by a Hitachi U-2001 spectrometer.

In vitro glycation of proteins

The methodology was based on that of Brownlee *et al*. (1986). Bovine serum albumin (BSA) (l0 mg/ml) was incubated with glucose (500 mM) in phosphate buffered-saline (PBS) (5 ml total volume, pH 7.4) or 0.1 M methylglyoxal, containing 0.02% sodium azide at 37 °C. All of the reagent and samples were sterilized by filtration through 0.2 µm membrane filters. The protein, the sugar and the prospective inhibitor were included in the mixture simultaneously. Aminoguanidine was used as an inhibitor positive control. Reactions without any inhibitor were also set up. Each solution was kept in the dark in a capped tube, incubation time was 15 days, and carried out by triplicate. The formation of AGEs was firstly assessed by the characteristic fluorescence (excitation wavelength of 370 nm and emission wave-length of 440 nm).

Percent inhibition was calculated as follows:

$$\textbf{Inhibition\%} = [1-(A_s-A_b)/(A_c-A_b)] \times 100$$

Where:

As = fluorescence of the incubated mixture with sample

A_c, A_b = are the fluorescence of the incubated mixture without sample as a positive control and the fluorescence of incubated mixture without sample as a blank control.

BSA-Methylglyoxal Assay

This assay was modified from on a published method (Peng *et al*., 2008). The assay evaluates the middle stage of protein glycation. BSA and methylglyoxal were dissolved in phosphate buffer (100 mM, pH 7.4)

to a concentration of 20 mg/ml and 60 mM, respectively. Compounds at concentration of 500 to 2000 µM were dissolved in the same phosphate buffer. 1 ml of the BSA solution was mixed with 1 ml of methylglyoxal solution and 1 ml of tested compounds. The mixture was incubated at 37 °C. Sodium azide (0.2 g/l) was used as an aseptic agent. Phosphate buffer was used as a blank. Aminoguanidine and phloroglucinol were used as positive controls. After seven days of incubation, fluorescence of the samples was measured using an excitation of 340 nm and an emission of 420 nm, respectively.
The % inhibition of AGE formation = [1 – (fluorescence of the test group/ fluorescence of the control group)] x 100%

Amadorin activity
Amadorin activity was determined using a post-Amadori screening assay (Khalifah *et al*., 1999). Lysozyme (10 mg/ml) was incubated with 0.5 M ribose in 0.1 M sodium phosphate buffer containing 3 mM sodium azide, pH 7.4 at 37 °C for 24 h. Unbound ribose was removed by dialysis against 4 1 of 0.1 M sodium phosphate buffer, Ph 7.4 at 4 °C for 48 h with 5-6 changes. Following dialysis, the protein concentration was determined using the Bio-Rad standard protein assay kit based on the Bradford dye-binding procedure (Bradford, 1976). Dialysed ribated lysozyme (10 mg/ml) was reincubated with 5-50 mM of compounds isolated and aminoguanidine in 0.1 M sodium phosphate buffer containing 3 mM sodium azide, pH 7.4 at 37 °C for 15 days.

Glycation of hemoglobin
Glassware was previously sterilized. The experiment was performed on a specially treated bench to avoid any possible contamination. Glucose (0.278 mg/dl), hemoglobin (30 mg/dl), isolated compound (5 mg/dl), and glutathione (0.5 mM) were dissolved in distilled-sterilized water. This hemoglobin solution was diluted with three times of water to prepare the negative control. Hemoglobin, glucose, and water were mixed at a ratio of 1:1:2 (v/v/v). Isolated compounds or glutathione was added instead of water to the positive control group. These mixtures were incubated for 5 days at 37 °C with continuous stirring (70 rpm). The amount of glycated hemoglobin (% GHb) was determined using an ion capture component set (IMx system, Abbott laboratories, USA). The amount of hemoglobin A_{lc} (% HbA_{lc}) was calculated by defining the equation used to convert IMx glycated hemoglobin (% GHb) to standardized percent of hemoglobin A_{lc} (% HbA_{lc}).

LDL oxidation measurement
Amphotericin B was dissolved in methanol first and then added to an LDL solution, with or without compound treatment, for final concentration of 5 and 10 µM of Amphotericin B. 1 ml of $CuS0_4$ (10 µM) was used to initiate LDL oxidation in 10 ml of an LDL solution sample. After incubating the LDL solution at 37 °C for 72 h, the method of Jain and Palmer (1997) was used to measure malondialdehyde (MDA) formation (nmol/mg LDL protein). Briefly, 0.2 ml LDL solution was suspended in 0.8 ml PBS. Then 0.5 ml trichloroacetic acid (TCA; 30%) was added. After vortexing and standing in ice for 2 h, samples were centrifuged at 1500 x *g* for 15 min. Supernatant (1 ml.) was mixed with 0.25 ml, thiobarbituric acid (TBA) (1%), and the mixture was heated in a boiling water bath for 15 min. The concentration of MDA-TBA complex was assayed at 532 nm.

The formation of conjugated diene (CD), a lipid oxidation product, in LDL also was determined according to the method described by Esterbauer *et al.* (1989). The lipid oxidation of an LDL solution containing 5 or 10 µM of each compound was initiated at 37 °C by 0.1 mM $CuCl_2$. Absorbance at 234 nm was continuously recorded for 60 min at 37 °C by a Hitachi U-2001spectrometer with a constant temperature recirculator. The lag phase, expressed in minutes, was defined as the period where no oxidation occurred. A longer lag phase indicated less CD formation.

In vitro glycation of LDL
LDL glycation was performed according to the method described in Li *et al.* (1996). Briefly, 50 mM glucose in PBS (pH 7.4) was added to an LDL solution with and without compound treatment. Sodium azide at 0.02% was used as antibiotic to prevent bacterial growth. This solution was sterile filtered, covered with N_2, and stored for 6 d at 37 ºC in the dark. After glycation, the solutions were dialyzed against PBS (20 ml, against 4 l) at 4 °C for 40 h. Then glycated LDL was separated from nonglycated LDL by applying a GlycoGel II column (Pierce, Rockford, IL), in which 500 µl LDL solution was loaded on the column, and glycated LDL was eluted with 2 ml, sorbitol buffer, pH 10.25. Neither copper nor any other oxidant was used for the

experiments on LDL glycation. The method of Duell *et al.* (1990) was used to measure LDL glycation level. LDL solution (200 µl) was mixed with 200 µl 4% $NaHC0_3$ and 200 µl 0.1% trinitrobenzoic acid. This mixture was flushed with N_2, sealed, and incubated at 37 °C in the dark. After 2 h, the absorbance at 340 nm was measured spectrophotometrically. The blank was a mixture of LDL and $NaHCO_3$ in PBS. LDL glycation is reported as relative reduction in the level of free ε-amino groups of L-lysine when compared with LDL solution in the absence of glucose. During LDL glycation, samples were treated with or without EDTA (0.5 mM), and LDL oxidation level was also determined.

Experimental animals

Study was conducted in CD1 mice weighting about 20-30 g. They were procured by the bioterium of the National School of Biological Sciences IPN and were housed in microlon boxes in a controlled environment (temperature 25 ± 2 ºC) with standard laboratory diet and *ad libitum* water. Animals were acclimatized for a period of three days in their new environment before initiating the experiment. Litter was renewed three times a week to ensure hygiene and maximum comfort of the animals. Ethical clearance for handling animals (NIH publication Nº 85-23 revised 1985) was observed.

***In vivo* glycation of proteins**

Experimental design

We used male mice (4-5 weeks age, 20-30 g) kept in wire-bottomed cages, and exposed to a 12-h light/dark cycle. The room temperature and humidity were maintained automatically at about 25 °C and 60%, respectively. These animals had *ad libitum* access to commercial food (Purina) and water. After several days of adaptation, the mice were randomly separated into normal control (n = 5) and diabetic groups. The diabetic groups were given an intraperitoneal (i.p.) injection of streptozotocin (45 mg/kg body weight in 10 mM citrate buffer (pH 4.5). Animals receiving an injection of citrate buffer were used as a normal control. After 10 days of the streptozotocin or vehicle injection, the blood samples were obtained from the tail vein between 10:00 and 11:00 am, avoiding the influence of food consumption and where used to determine glucose levels. Mice with a blood glucose level higher than 300 mg/dl were used as diabetic mice: and randomly divided into five experimental groups. Diabetic animals were treated orally with the tree compounds dissolved in water at doses of 15 mg/kg/day by oral gavage while their control group was only given water. Fourteen days later, after renal perfusion through the renal artery with ice-cold physiological saline, the kidneys were removed from each mouse.

Serum parameters

Serum glycosylated protein was measured by the thiobarbituric acid assay of McFarland *et al.* (1979) in which non-enzymatically bound glucose is released as 5-hydroxymethylfurfural and quantitated colorimetrically.

AGE Level in kidney

The renal AGEs level was determined by the method of Nakagawa (1993). In brief, minced kidney tissue was dilapidated with chloroform and methanol (2: 1, v/v) overnight. After washing, the tissue was homogenized in 0.1 N NaOH, followed by centrifugation at 8000 x g for 15 min at 4 °C. The amounts of AGEs in these alkali-soluble samples were determined by measuring the fluorescence at an emission wavelength of 440 nm and an excitation wave length of 370 nm. A native BSA preparation (l mg/ml of 0.1 N NaOH) was used as a standard, and its fluorescence intensity was defined as one unit of fluorescence. The fluorescence values of samples were measured at a protein concentration of 1mg/ml and expressed in arbitrary units (AU).

Glycation of tail tendon collagen

Tendons separated from tails of experimental mice were washed thoroughly in saline solution at 4 ºC. Acid hydrolysis of the tendons was carried out at 121 ºC for 4 h. Hydroxyproline was estimated in hydrolysed tendon collagen samples according to the method of Woessner (1961). Collagen content in the mouse tail tendon was expressed in mg collagen/100 mg tissue assuming that collagen weights 7.46 times hydroxyproline. Extent of collagen glycation in tail tendon was assessed by phenol-sulfuric acid method (Rao and Pattabiraman, 1989) and expressed as, µg of glucose/mg collagen. Clean tendons (50 mg, wet weight) were digested in freshly prepared pepsin solution (1 mg/ml in 0.5 M acetic acid, 5 ml) for 24 h at 37 ºC to determine the amount of pepsin soluble collagen (Golub *et al*., 1978). Samples of pepsin-digested collagen (0.25 ml) were mixed with 2.75 ml of 200 mM phosphate buffer (pH 7.5). Collagen linked fluorescence was quantified at 365 nm excitation and

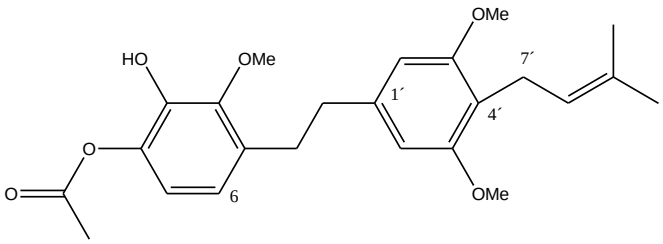
416 nm emission, relative to the standard quinine sulfate solution (1 µg/ml) and expressed as AU/mg collagen (Stefek *et al*., 2000).

Statistical Analysis

Data are presented as means ± S.E.M. The effect of isolated compounds **1** to **3** on each parameter was examined using the one way Analysis of Variance. Individual differences among groups were analyzed by Dunnett's test and significance was accepted at $p < 0.05$.

RESULTS AND DISCUSION

Repeated chromatography of the chloroform residue from bulbs of *P. michuacana* led to the isolation and characterization of α-α´-dihydro, 3´,5´,2-trimethoxy-3-hydroxy-4-acetyl-4´-isopentenylstilbene (**1**) 4,6,7-trihydroxy-2-methoxy-8-(methylbut-2-enyl phenanthren)-1-1´-4´,6´,7´-trihydroxy-2´-methoxy-8´-(methylbut-2´-enyl) phenanthrene (**2**) and gigantol (**3**) (Figure 1). The structures were elucidated based on 1-dimensional (^{1}H- and ^{13}C-NMR) and 2-dimensional NMR heteronuclear multiple bonding connectivity (HMBC) spectral data analysis and by comparison with published spectral data. We have previously reported the elucidation of the structure and their antioxidant effect on DPPH and ABTS radical scavenging and anti-lipid peroxidation activities (Perez *et al*., 2010b).



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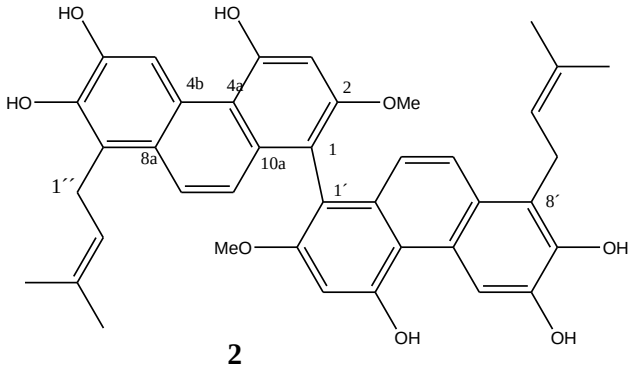

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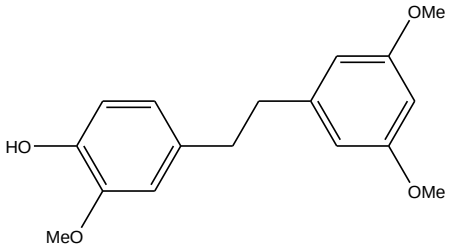

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Figure 1
Chemical structures of compounds 1-3 isolated from *Prosthechea michuacana*

Table 1
The inhibitory effects of chloroform extract from *P. michuacana* (PM), compounds 1, 2, 3 and aminoguanidine on the formation of advanced glycation end products (AGEs), *in vitro* induced by glucose and methylglyoxal

Inducer	Treatment	AGEs IC_{50}
Glucose	Chloroform extract (PM)	74.6 µg/ml
	1	24.5 µM
	2	71.6 µM
	3	28.7 µM
	Aminoguanidine	57.3 µM
Methylglyoxal	**1**	1013.7 µM
	2	1578.9 µM
	3	1018.4 µM
	Aminoguanidine	910.9 µM

In order to determine the inhibitory effect of the three isolated compounds on AGEs formation, several assay methods have been proposed including assays based on inhibition of specific fluorescence generated during the course of glycation and AGEs formation, and assays based on the inhibition of AGEs-protein cross-linking. Among these, stilbene **1** and **3** exhibited the higher inhibitory activity against AGEs formation after incubation at 37° C for 15 days, with an IC_{50} value of 24.5 and 28.7 µM respectively (Table 1). Methylglyoxal-mediated protein glycation inhibition was evaluated for isolated **1** to **3** (Table 1) which exhibited a substantial activity, although still less effective than that of aminoguanidine. The inhibitory effect of the three compounds and aminoguanidine had IC_{50} values of 1013.7 and 1018.4 and 910.9 µM, respectively. Inter and intra molecular cross linking with collagen is formed as a result of glycation which is responsible for resistance to pepsin digestion. Treatment with isolated compounds and glibenclamide reversed these parameters with respect to the diabetic control. Treatment with isolated compounds and glibenclamide significantly reduced the levels of collagen linked fluorescence, which is in agreement with the *in vitro* BSA glycation study. Solubility pattern was also restored, with a relative increase in pepsin soluble collagen. These changes indicated a reduction in cross-linking of collagen proteins in isolated treated diabetic animals.

The effect of 1-**3** on the formation of AGEs in the GK-peptide/ribose system was then studied. This test is used to evaluate the ability of the extract to inhibit the crosslinking of GK-peptide in the presence of ribose. Compounds **1**, **2**, **3** and aminoguanidine were found to inhibit the formation of AGE (fluorescence) after 15 days of incubation, as shown in Table 2.

Glucose reacts nonenzymatically with the NH_2-terminal amino group of the β chain of human hemoglobin (HbA; $\alpha_2\beta_2$) to form an aldimine linkage, which subsequently undergoes an Amadori rearrangement to form the more stable ketoamine linkage, resulting in the slow formation of HbA_{1c}. Table 3 shows the amount of glycated hemoglobin (% GHb). When hemoglobin was used alone (NC), the amount of glycated hemoglobin was 8.9%. This noticeably increased with the addition of glucose to a 27.6% (PC). Nonetheless, it decrease significantly with the treatment of **1**, **2** and **3** and dropped further with the treatment of glutathione. The amount of hemoglobin A_{1c} (%HbA_{1c}), corresponds to a specific sub-fraction of glycated hemoglobin, it is lower than the amount of glycated hemoglobin. However, it showed a similar tendency in the percentage of glycation. This result indicates that compounds have the most potent glycation inhibition at the early stage of protein glycation at a concentration, of 5 mg/ml. These compounds can effectively prevent HbA_{1C} formation.

Glycosylated protein levels in blood serum of diabetic mice were significantly higher than those of control mice as expected. At the end of the experiment after treatment with the isolated compounds, glycosylated protein, in diabetic mice were statistically decreased (Table 3).

Table 2
Percentage inhibition of crosslinked advanced glycation endproducts following reincubation of ribated lysozyme

Treatment (mM)	Inhibition (%)
1	
25	39
50	57
2	
25	27
50	48
3	
25	34
50	52
Aminoguanidine	
50	60

Table 3
The inhibitory effects of chloroform extract and stilbenoids 1, 2 and 3 on glycosylated protein and glycated hemoglobin (GHb and HbA_{1c})

Groups	GHb	HbA_{1c}	Glycosylated protein
(nmol/mg protein)			
Negative control	8.9 ± 0.09	7.8 ± 0.2	15.2 ± 0.23[a]
Positive control	27.6 ± 0.12	18.6 ± 0.4	23.6 ± 0.04
Chloroform extract	20.4 ± 0.16[a]	15.9 ± 0.6[a]	18.7 ± 0.12[a]
1	9.8 ± 0.17[a]	10.0 ± 0.5[a]	19.5 ± 0.14[a]
2	13.6 ± 0.09[a]	15.0 ± 0.3[a]	17.2 ± 0.51[a]
3	10.1 ± 0.18[a]	12.7 ± 0.4[a]	18.1 ± 0.07[a]
Glutathione	8.1 ± 0.08[a]	9.0 ± 1.1[a]	-
Aminoguanidine	-	-	20.1 ± 0.07[a]

Negative Control: Incubation with hemoglobin (30 mg/ml), positive control: Incubation with hemoglobin (30 mg/ml) + glucose (0.278 mM), chloroform extract: Incubation with hemoglobin (30 mg/ dI) + glucose (0.278 mM) + chloroform extract (20 mg/ ml), stilbenoids: Incubation with hemoglobin (30 mg/ dl) + glucose (0.278 mM) + stilbenoids (5 mg/ml), glutathione: Incubation with hemoglobin (3 mg/ dl) + glucose (2.7 mM) + glutathion (0.5 mM). Data as expressed as ± SD; [a]$P < 0.05$ vs positive control values.

Amphotericin B treatments at 5 and 10 µM significantly increased LDL oxidation levels as by determined MDA formation (Table 4, $P < 0.05$). However, the presence of the three isolated compounds at 5 µM significantly reduced 10 µM amphotericin B-induced LDL oxidation (Table 4, $P < 0.05$). Compound, **2** was to be most effective agent.

Table 4
The prooxidant effect of amphotericin B (AB) at 5 and 10 µM, and the antioxidant protection of 5 µM 1, 2 and 3 against 10 µM, AB-induced malondialdehyde (MDA) formation (nmol/mg LDL protein) after a 48-h incubation at 37° C.

Groups	MDA formation (nmol/mg LDL protein)
Control	13.26 ± 2.16
AB 5 µM	22.56 ± 5.72[a]
AB 10 µM	34.29 ± 2.34[a]
AB 10 µM + **1**(5 µM)	32.56 ± 1.89[a]
AB 10 µM + **2**(5 µM)	28.42 ± 3.27[a]
AB 10 µM + **3**(5 µM)	30.19 ± 5.81[a]

Values are expressed as Mean ± SD, [a]Significantly ($P < 0.05$) different from control, where the significance was performed by Oneway ANOVA followed by post hoc Dunnett´s test

LDL treated with 50 mM glucose and EDTA significantly increased glycation level (Table 5, $P < 0.05$). Under EDTA protection, the presence of the three bioactives at 5 µM significantly reduced LDL glycation, and the most powerful antiglycative agent was **1** ($P < 0.05$). On the other hand, both LDL oxidation and LDL glycation significantly increased when LDL was treated with 50 mM glucose without EDTA protection. Although the presence of three isolated at 5 and 10 µM significantly reduced both LDL oxidation and glycation when compared with controls ($P < 0.05$), the most antioxidative and antiglycative agent was **1**.

Table 5
Protective effect of 5 µM, of 1, 2 and 3 on LDL against 50 mM glucose-induced glycation and oxidation with or without 0.5 mM EDTA treatment

	With EDTA		Without EDTA	
Treatment	Glycation	Oxidation	Glycation	Oxidation
LDL	2.8 ± 0.17	3.6 ± 0.63	3.8 ± 0.87	21.03 ± 2.10
LDL+glucose	17.2 ± 3.32[a]	4.3 ± 1.56[a]	22.65 ± 3.45[a]	58.79 ± 3.86[a]
1(5 µM)	4.6 ± 0.98[b]	2.9 ± 0.98[a,b]	13.86 ± 2.74[a,b]	41.78 ± 2.87[a,b]
2(5 µM)	10.4 ± 2.76[a,b]	3.7 ± 0.62[b]	18.59 ± 5.03[a,b]	43.32 ± 4.39[a,b]
3(5 µM)	6.3 ± 1.80[a,b]	3.2 ± 0.87[a,b]	15.14 ± 2.96[a,b]	46.50 ± 4.17[a,b]

Values are expressed as Mean ± SD (n = 6), [a]Significantly ($P < 0.05$) different from LDL group. [b]Significantly ($P < 0.05$) different from LDL+glucose, where the significance was performed by Oneway ANOVA followed by post hoc Dunnett´s test

Advanced AGEs are a heterogeneous group of products arising from the non-enzymatic glycation and oxidation of proteins and lipids (Duell *et al.*, 1990). They accumulate in diverse biological settings, such as diabetes, inflammation, renal failure, and aging (Ahmed, 2005). A number of natural and synthetic compounds have been proposed, discovered, or are currently being used as AGEs inhibitors (Dukic-Stefanovic *et al.*, 2001). The AGEs inhibitors, such as pyridoxamine, aminoguanidine and LR90, have been shown to prevent the development of complications in experimental diabetes (Degenhardt *et al.*, 2002). In this study, PM inhibited advanced glycation end-

products-BSA formation *in vitro* and was less effective than the AGEs inhibitor aminoguanidine.

Increased glycation during hyperglycaemia can cause intra or inter molecular cross-linking of proteins as they accumulate AGEs. Numerous studies have reported that buildup of cross-linked AGEs on long-lived proteins may underlie the development of health complications in subjects affected by diabetes or because of ageing (Ahmed, 2005). Furthermore, levels of serum AGEs reflect the severity of these complications whereas therapeutic interventions aimed at reducing AGEs can inhibit or delay their progression (Monnier, 2003). In this study we found that stilbenoids **1** and **3** inhibited formation of methylglyoxal derived AGEs in a bovine serum-albumin-methylglyoxal system, and may also act by blocking conversion of dicarbonyl intermediates to AGEs. This effect may be due to the unsubstituted carbons of compounds **1** and **3** at their position 6 on the A-ring, which is the best active sites for to trap reactive dicarbonyl species. This was consistent with previous finding by Sang et al (2007).

Furthermore, ours results show that isolated compounds possesses Amadorin activity. Exposure of lysozyme to ribose for a period of 24 h generates glycated protein rich in Amadori but not advanced glycation adducts (Khalifah *et al*., 1999). Reincubation of this ribated lysozyme in the absence of sugar generates cross-linked AGEs as reported previously (Liggins and Furth, 1996) but their formation inhibited in the presence of PM. In addition to its antioxidant properties previously reported (Perez *et al*., 2010), the compounds isolated from PM could react with carbonyl groups from reducing sugars, Amadori adducts and dicarbonyl intermediates therefore blocking their conversion to advanced glycation end-products. Dicarbonyl intermediates such as methylglyoxal have received considerable attention as mediators of advanced glycation end-product formation and are known to react with lysine, arginine and cysteine residues in proteins to form glycosylamine protein crosslinks (Frye *et al*., 1998). Reincubation of dialyzed ribated lysozyme generated cross-linked advanced glycation end-products that were inhibited in the presence of increasing concentrations of analyzed compounds and aminoguanidine. All compounds have Amadorin activity and inhibit cross-linked advanced glycation end-products to concentrations of 5-50 mM.

Amphotericin B, with a great affinity to the lipid part of the LDL particle, could extract cholesterol from LDL and sensitize the modified LDL to oxidation. The compounds in this study exhibited marked antioxidant protection against amphotericin B induced oxidation (Table 3). Nonenzymatic glycation of LDL is accompanied by oxidative, radical-generating reactions (Menzel *et al*., 1997). In the presence of EDTA, oxidation was not responsible for the observed glycation; in this instance, glycation may be due simply to the interaction between LDL protein and glucose. These properties may allow them to exhibit a great affinity to glucose and or LDL protein.

Kidney weight, renal AGEs and mitochondrial thiobarbituric acid-reactive substance was very elevated in diabetic mice compared to the control group (Table 6). These levels were reduced to almost in range values by the administration of the different tested compounds. The mitochondrial thiobarbituric acid-reactive substance was increased to 2.09 mmol/mg protein compared with the 1.81 mmol/mg protein of the control mice. Remarkably, levels in groups treated with isolated were significantly reduced. The level of glucose in the diabetic control group increased during the period of the experiment and the administration of isolated did not have an effect on it. Symptoms in diabetic animals are increased kidney lipid peroxidation (TBARS), reduction in antioxidant defense and increase in renal AGE. This was in agreement with the present study results that confirmed by Maillard-type fluorescent measurement, renal AGEs accumulation in streptozotocin-induced diabetic mice. For this reasons, we first assessed the effect of analyzed compounds on renal AGE accumulation and thiobarbituric acid reactive substance. Even though, a 10 days experimental period is not enough time to observe structural modifications in kidneys, it is important to prevent diabetic renal damage from the early stage.

Collagen was measured in mice tail tendon after acid hydrolysis. Diabetic mice showed a significant increase in tail tendon collagen, collagen glycation and collagen linked f1uorescence and reduction in pepsin soluble collagen (Table 7). Treatment with **1** to **3** compounds and the control drug, glibenclamide, reversed these effects, indicating a potential role in preserving collagen structure and function during the pathogenesis of diabetes. The effect of stilbenoids at doses of 15 mg/kg was statistically ($p < 0.05$) comparable with that of glibenclamide.

CONCLUSIONS

In summary, the data presented here clearly indicates that *Prosthechea michuacana* extract possesses *in vitro* inhibitory activity against AGEs formation. Ultimately, a strong inhibitory compounds on glycation were isolated from a chloroform extract of the bulbs of orchid *P. michuacana* and identified as α-α´-dihydro, 3´,5´,2-trimethoxy-3-hydroxy-4-acetyl-4´-isopentenyl stilbene, 4,6,7-trihydroxy-2-methoxy-8-(methylbut-2-enylphenan-thren-1-1´-4´,6´,7´-trihydroxy-2´-methoxy-8´-(methylbut-2´-enyl)-phenan-threne and gigantol. Isolated compounds possessed antioxidant, Amadory activities and inhibitory activity on AGEs formation. In streptozotocin-induced diabetic rats, to prevent and/or delay the onset of diabetic renal damage. Therefore, stilbenoids and phenantrenes from *P. michuacana* could be a candidate for use in studies looking at the effects of natural herbal complement in the prevention of diabetes complications, since it possesses both antioxidant and antyglycation activities.

Table 6
Effect of 1, 2, 3 on thiobarbituric acid-reactive substances in mitochondrial, renal weight and AGE levels

Groups	Mitocondrial-TBA (mmol/mg protein)	Renal Weight (g)	AGE (AU)
Normoglucemic	1.81 ± 0.039[a]	0.73 ± 0.067[a]	15.98 ± 3.12[a]
Diabetic	2.09 ± 0.026	1.08 ± 0.018	24.25 ± 3.90
1	1.87 ± 0.012[a]	0.96 ± 0.064[a]	13.10 ± 3.01[a]
2	1.95 ± 0.094[a]	1.0 ± 0.087[a]	16.23 ± 4.21[a]
3	1.85 ± 0.039[a]	0.95 ± 0.072[a]	13.26 ± 3.48[a]
Aminoguanidine	1.80 ± 0.058[a]	0.97 ± 0.048[a]	12.87 ± 2.99[a]

Data as expressed as ± SD; [a]P < 0.05 vs. diabetic control values.

Table 7
Effect of 1, 2, 3 on the glycation of tail tendon collagen

Treatment	Total collagen (mg/100 mg tendon)	Pepsin digested (mg/100 mg tendon)	Collagen glycation (µg glucose/collagen)	Fluorescence (AU/mg collagen)
Normal	37.6 ± 1.86	3.46 ± 0.043	2.98 ± 0.65	2.85 ± 0.054
Diabetic	75.9 ± 0.98	1.47 ± 0.036	14.57 ± 0.79	24.63 ± 0.96
Diabetic +**1**	48.3 ± 1.43 [ab]	2.13 ± 0.028[ab]	8.20 ± 0.32 [ab]	17.65 ± 0.54 [ab]
Diabetic + **2**	66.4 ± 1.78 [ab]	1.74 ± 0.019[ab]	10.69 ± 0.57 [ab]	20.13 ± 1.03 [ab]
Diabetic + **3**	48.9 ± 2.06[ab]	2.16 ± 0.026 [ab]	8.53 ± 0.62 [ab]	17.70 ± 0.79[ab]
Diabetic + Glibenclamide	47.1 ± 1.87[ab]	2.19 ± 0.020 [ab]	8.09 ± 0.14 [ab]	17.21 ± 0.72 [ab]

Data as expressed as ± SD; n=5; [a] vs normal control; [b] vs diabetic control. Concentration of analyzed compounds: 15 mg/kg pde peso

REFERENCES

Ahmed N. 2005. Advanced glycation endproducts-role in pathology of diabetic complications. **Diabetes Res Clin Pract** 67: 3 - 21.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal Biochem** 7: 248 - 254.

Brownlee M. 1995. Advanced protein glycosylation in diabetes and aging. **Annu Rev Med** 46: 223 - 234.

Brownlee M, Vlassara H, Kooney A, Ulrich P, Cerami A. 1986. Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. **Science** 232: 1629 - 1632.

Degenhardt TP, Alderson NL, Arrington DA, Beattie RJ, Basgen Steffes MW, Thorpe SR, Baynes JW. 2002. Pyridoxarnine in early renal disease and dyslipidemia in the streptozotocin-diabetic rat. **Kidney Int** 6l: 939 - 950.

Dukic-Stefanovic S, Schinzel R, Riederer P, Munich G. 2001. AGES in brain ageing: AGE-inhibitors as neuroprotective and anti-dernentia drugs. **Biogerontology** 2: 19 - 34.

Duell PB, Oram JF, Biermann EL. 1990. Nonenzymatic glycation of HDL resulting in inhibition of high-affinity binding to cultured human fibroblasts. **Diabetes** 39: 1257 - 1263.

Esterbauer H, Striegl G, Puhl H, Rotheneder M. 1989. Continuous monitoring of *in vitro* oxidation of human low Density Lipoprotein. **Free Radic Res Commun** 6: 67 - 75.

Flores MA, Manzanero GM. 1988. Los tipos de vegetación del estado de Oaxaca. **Soc Nat Oaxaca** 25: 7 - 45.

Frye EB, Degenhardt TP, Thorpe SR. 1998. Role of the Maillard reaction in aging of tissue proteins. **J Biol Chem** 273: 18714 - 18719.

Golub IM, Greenwald RA, Zebrowski EJ, Ramamurthy K. 1978. The effect of experimental diabetes on the molecular characteristics of soluble rat collagen. **Biochim Biophys Acta** 534: 73 - 81.

Jain SK, Palmer M. 1997. The effect of oxygen radical metabolites and vitamin E on glycosylation of proteins. **Free Radic Biol Med** 22: 593 - 596.

Khalifah RG, Baynes JW, Hudson BG. 1999. Amadorins: novel post-Amadori inhibitors of advanced glycation reactions. **Biochem Biophys Res Commun** 257: 251 - 258.

Kune S, Takeya M, Mori T, Araki N, Susuki H, Horiuchi S, Kodama T. 1978. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. **Anal Biochem** 86: 271 - 278.

Li D, Devaraj S, Fuller C, Bucala R, Jialal I. 1996. Effect of α-tocopherol on LDL oxidation and glycation: *In vitro* and *in vivo* studies. **J Lipid Res** 37: 1978 - 1986.

Liggins J, Furth AJ. 1996. Mixed dirners fonned by cross-Iinking of native and glycated proteins in the absence of free sugar. **Biochem Biophys Res Commun** 219: 186 - 190.

McFarland KF, Catalano EW, Day JF, Thorpe SR, Baynes JW. 1979. Nonenzymatic glucosylation of serum proteins in diabetes mellitus. **Diabetes** 28: 1011 - 1014.

Menzel EJ, Sobal G, Staudinger A. 1997. The role of oxidative stress in the long- term glycation of LDL, **Biofactors** 6: 111 - 124.

Monnier VM. 2003. Intervention against the Maillard reaction in *vivo.* **Arch Biochem Biophys** 419: 1 - 15.

Nakagawa S. 1993. Immunochemical detection of advanced glycation end products in lens crystallins from streptozotozin-induced diabetic rats. **Diabetes** 42: 345 - 350.

Peng X, Zheng Z, Cheng KW, Shan F, Ren GX, Chen F, Wang M. 2008. Inhibitory effect of mung bean extract and its constituents vitexin and isovitexin on the formation of advanced glycation endproducts. **Food Chem** 106: 475 - 481.

Perez GRM, Vargas RS. 2009a. Hepatoprotective and inhibition of oxidative stress in liver of *Prosthechea michuacana*. **Rec Nat Prod** 3: 46 - 51.

Perez GRM, Vargas SR. 2009b. Anti-inflammatory and wound healing potential of *Prosthechea michuacana* in rats. **Phcog Mag** 4: 219 - 225.

Perez GRM. 2010. Orchids. A review of uses in traditional medicine, its phytochemistry and pharmacology. **J Med Plants Res** 4: 592 - 638.

Perez GRM, Gomez YGG, Bautista ER. 2010a. Nephroprotective activity of *Prosthechea michuacana* against cisplatin-induced acute renal failure in rats. **J Med Food** 13: 911 - 916.

Perez GRM, Neira GAM, Garcia EB, Lugardo S. 2010b. Studies on the constituents of the orchid *Prosthechea michuacana* bulbs and antioxidant activity. **Chem Nat Compounds** 46: 554 - 561.

Perez GRM, Hoyos VC. 2011. Effect of flavonoids from *Prosthechea michuacana* on carbon tetrachloride- induced acute hepatotoxicity in rats. **Pharm Biol** 49: 1121 - 1127.

Perez GRM, Vadillo CH. 2011. Anti-diabetic activity of hexane extract from *Prosthechea michuacana* in streptozotocin-induced diabetic rats. **Bol Latinoam Caribe Plants Med Aromat** 10: 570 - 580.

Rahbar S, Figarola, J. 2002. Inhibitors and breakers of advanced glycation end-products (AGEs). A review. **Curr Med Chem Immunol Endrocr Metab Agents** 2: 135 - 161.

Rao P, Pattabiraman TN. 1989. Reevaluation of the phenol sulphuric acid reaction for the estimation of hexoses and pentoses. **Anal Biochem**181: 18 - 22.

Sang S, Shao X, Bai N, Lo CY, Yang CS, Ho CT. 2007. Tea polyphenol (-)-epigallocatechin-3-gallate: a new trapping agent of reactive dicarbonyl species. **Chem Res Toxicol** 20: 1862 - 1870.

Stefek M, Gajdosik A Gajdosikova A, Krizanova L. 2000. p-Dimethyl-aminobenzaldehyde reactive substances in tail tendon collagen of streptozotocin-diabetic rats: temporal relation to biomechanical and advanced glycation endproduct (AGE)-related f1uorescence. **Biochim Biophys Acta** 1502: 398 - 404.

Vargas RS, Perez GRM. 2009. Relaxant and antispasmodic effects of extracts of orchid *Prosthechea michuacana* on isolated guinea pig ileum. **J Nat Med** 63: 65 - 68.

Vitek MP, Bhattacharya K, Glendening JM, Stopa E, Vlassara H, Bucala R, Manogue K. 1994. Advanced glycation end-products contribute to amyloidosis in Alzheimer´s disease. **Proc Natl Acad Sci** 91: 4766 - 4770.

Woessner JE. 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. **Arch Biochem Biophys** 93: 440 - 447.